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Title page

Oseltamivir (Tamiflu™) is a substrate of PEPT1

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Running title page.

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Oseltamivir is a substrate of PEPT1

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PEPT1, peptide transporter1; P-gp, P-glycoprotein; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; CL_{tot}, total clearance; HPLC, high-performance liquid chromatography; TEER, transepithelial electrical resistance; FBS, fetal bovine serum; Gly-Sar, glycyl-sarcosine; BNPP, bis(4-nitrophenyl) phosphate; BSA, bovine serum albumin; C_{max}, The maximum plasma concentration; T_{max}, time to C_{max}; $AUC_{0\text{-}6hr}$, area under the plasma concentration-time curve from time 0 to 6 hr; CES, human carboxylesterase

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ABSTRACT

Oseltamivir, an ester-type prodrug of the neuraminidase inhibitor Ro 64-0802, has been developed for the treatment of A and B strains of the influenza virus, but has neuropsychiatric and other side effects. In this study, we characterized the transport across intestinal epithelial cells and the absorption of oseltamivir in rats. Uptake by Caco-2 cells (human carcinoma cell line) and HeLa cells transfected with peptide transporter1 (HeLa/PEPT1) was time- and temperature-dependent, and was inhibited by typical PEPT1 inhibitors such as glycyl-sarcosine (Gly-Sar). The uptake by Caco-2 cells and HeLa/PEPT1 was saturable, with similar K_m values. Oseltamivir absorption in adult rats was greatly reduced by simultaneous administration of milk, casein or Gly-Sar. Further, the plasma and brain concentrations of oseltamivir were higher in fasting than in non-fasting rats after oral administration. These results suggest that oseltamivir is a substrate of PEPT1, and that PEPT1 is involved in its intestinal absorption.

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INTRODUCTION

Various transporters are expressed on apical and basolateral membranes of intestinal epithelial cells, serving to take up nutrients and to excrete xenobiotics into the lumen. Influx transporters are able to accept nutrients and also various drugs as substrates. In particular, peptide transporter1 (PEPT1, SLC15A1), localized at brush-border membranes of human small intestine (Saito et al., 1995), plays important roles in the absorption of not only di/tri-peptides (Tamai et al., 1994), but also peptide-mimetic compounds, such as orally administered beta-lactam antibiotics (Ganapathy et al., 1995; Sai et al., 1996) and the anticancer agent bestatin (Tomita et al., 1990; Inui et al., 1992). Recently, several researchers have found that intestinal PEPT1 can transport L-valine ester prodrugs, such as valacyclovir and valgancyclovir (Balimane et al., 1998; Han et al., 1998; Sugawara et al., 2000). Therefore, such structural modification of drugs may result in increased intestinal absorption, mediated by PEPT1.

Oseltamivir phosphate (oseltamivir), manufactured under the trade name Tamiflu™ as an ester-type prodrug of the neuraminidase inhibitor Ro 64-0802, has been developed for the treatment of A and B strains of the influenza virus. This drug has been reported to be associated with neuropsychiatric side effects (http://www.fda.gov/cder/drug/infopage/tamiflu/QA20051117.htm and http://www.mhlw.go.jp/ english/index.html), which are likely to be caused by distribution of oseltamivir and/or its metabolite(s) to the central nervous system. Recently, we examined the possible role of P-glycoprotein (P-gp) as the determinant of brain distribution of oseltamivir and Ro 64-0802 both in vitro using LLC-GA5-COL150 cells, which over-express human MDR1 P-gp on the apical membrane, and in vivo using mdr1a/1b knockout mice (Morimoto et al., 2008). The permeability of oseltamivir in the basolateral-to-apical direction was significantly greater than that in the opposite direction. The brain distribution of oseltamivir was increased in mdr1a/1b knockout mice compared with wild-type mice. In contrast, negligible transport of Ro 64-0802 by P-gp was

observed in both in vitro and in vivo studies. These results demonstrated that oseltamivir, but not Ro 64-0802, was a substrate of P-gp. Accordingly, low levels of P-gp activity or drug-drug interactions at P-gp may lead to enhanced brain accumulation of oseltamivir, and this in turn may account for the central nervous system effects of oseltamivir observed in some patients (Morimoto et al., 2008). During that research, we noticed that increased plasma concentration and toxicity were observed in fasted infant rats compared with non-fasted ones. Therefore, we speculated that transporters involved in the uptake of food components also take part in the absorption of oseltamivir.

The purpose of the present study was to characterize the transport of oseltamivir across intestinal epithelial cells. We first examined whether oseltamivir is a substrate of PEPT1 by using a human carcinoma cell line, Caco-2 cells, and HeLa cells stably expressing human PEPT1 (HeLa/PEPT1). A rat in vivo study was then conducted to confirm involvement of PEPT1 in oseltamivir absorption in the small intestine. Our findings indicate that a drug-food interaction with potential clinical significance is likely to occur between oseltamivir and milk.

METHODS

Chemicals and animals

Oseltamivir phosphate was purchased from Sequoia Research Products (Pangbourne, UK). Ro 64-0802 was biologically synthesized from oseltamivir using porcine liver esterase (Sigma, St Louis, MO) as described previously (Morimoto et al., 2008). The human colon adenocarcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (Rockville, MD, USA). HeLa/PEPT1 and HeLa transfected with vector alone (mock) were established as described previously (Nakanishi et al., 2000). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids, penicillin, streptomycin, gentamycin, and Hanks' balanced salt solution (HBSS) were all from Invitrogen Corp. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from ICN

Biomedicals, Inc. (Osaka, Japan), and type-I collagen solution was from Nitta Gelatin (Osaka, Japan). Glycyl-sarcosine (Gly-Sar), bis(4-nitrophenyl) phosphate (BNPP) and bovine serum albumin (BSA) were from SIGMA (St. Louis, MO). The protein assay kit was purchased from Bio-Rad Laboratories Inc. (Hercules, CA). All other chemicals and solvents were commercial products of analytical, HPLC or LC/MS grade as appropriate.

The animal study was performed according to the Guidelines for the Care and Use of Laboratory Animals at the Takasaki University of Health and Welfare and approved by the Committee of Ethics of Animal Experimentation of the university. One- and eight- week-old Wistar rats were purchased from SLC Japan (Hamamatsu, Japan)

Cell culture and cellular quality assessment

Caco-2 cells were cultured in DMEM containing 10% FBS, 1% nonessential amino acids, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 25 μg/mL gentamycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells at passage number from 60 to 80 were used. For the transport study, Caco-2 cells were routinely grown to confluence in 75-cm^2 tissue culture dishes and seeded into Transwell inserts coated with type-I collagen (pore size: 0.4 μm; diameter: 12 mm, Costar, Cambridge, MA, USA). The cells were seeded at a density of 2 x 10^5 cells/cm^2 and monolayers were formed after culturing for 2 weeks. The integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance (TEER) with Millicell-ERS equipment (Nihon Millipore, Tokyo, Japan). Monolayers with a TEER of more than 300 Ω•cm^2 were used for the transepithelial transport experiments. These monolayers were also used for permeation studies, as previously reported (Kobayashi et al., 2008). TEER of the monolayers was measured before and after each transport experiment. For the uptake study, Caco-2 cells were seeded at a density of 2 x 10^5 cells/cm^2 on multiwell dishes (Nunc, Naperville, IL) coated with collagen. Cells were grown for 7 days for the

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uptake experiment (Kimoto et al., 2007).

HeLa/PEPT1 and mock cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, and 1 mg/mL geneticin (G418) as described previously (Nakanishi et al., 2000). For the uptake assay, each cell line was seeded on eight-well plates (Nunc, Naperville, IL) and cultured for 3 days.

Transcellular transport and uptake experiments

In the transcellular transport study, monolayers of Caco-2 cells were gently rinsed twice with HBSS [136.9 mM NaCl, 5.37 mM KCl, 5.55 mM D-glucose, 1.258 mM $CaCl_2$, 0.441 mM KH_2PO_4, 0.811 mM $MgSO_4$, 0.337 mM Na_2HPO_4, 4.047 mM $NaHCO_3$, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) for pH 7.4 or 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) for pH 6.0] and left to equilibrate in the same solution for 20 min at 37°C. To measure the apical-to-basolateral permeability, 1.5 mL of HBSS (pH 7.4, 37°C) was added to the basolateral chamber of the Transwell insert and then 0.5 mL of the test solution (pH 6.0, 37°C) containing oseltamivir was added to the apical side. After the desired incubation time at 37°C, the basolateral solution was collected, and replaced with an equal volume of HBSS. To investigate the effect of esterase inhibitor treatment of Caco-2 cells on the permeability of oseltamivir in the apical-to-basolateral direction, Caco-2 cells were preincubated with 200 μM BNPP, a specific carboxylesterase inhibitor (Block et al., 1978; Mentlein et al., 1988) at 37°C for 45 min. We also examined the influence of permeability of oseltamivir across a Caco-2 cell monolayer in the presence of 10 μM verapamil, an inhibitor of P-gp (Ogihara et al., 2004).

In the case of the uptake study, Caco-2 cells grown on the multidishes were washed with 2 mL of HBSS and preincubated for 20 min. After preincubation, HBSS (300 μL) containing oseltamivir was added to initiate uptake. The cells were incubated at 37°C for a designated time, and

then washed five times with 2 mL of ice-cold HBSS to terminate the uptake.

Uptake study with HeLa cells was also performed at 37°C in HBSS adjusted to pH 6.0. Cultured cells were washed and preincubated in the buffer without oseltamivir or Ro 64-0802 for 10 min at 37°C. The uptake was initiated by adding HBSS (300 μL) containing oseltamivir or Ro 64-0802. After incubation for designated times at 37°C, the experiment was terminated by removing the medium, followed by washing five times with 2 mL of ice-cold HBSS. For quantitation of the drug taken up by the cells, the cells were suspended in acetonitlile/methanol (25/75; vol/vol), collected with a cell scraper (Asahi Techno Glass Corporation Co. Ltd., Chiba, Japan), and then sonicated for 30 min, followed by centrifugation at 12000 rpm and 4˚C for 10 min. The supernatant was evaporated under centrifugal evaporator at 30˚C. The residue was dissolved in 0.1 mL of 10 mM ammonium acetate buffer (pH7.0) and filtered by passing through a 0.45 μm pore size membrane filter (Millipore Corporation, Billerica MA, USA). The supernatants were then subjected to LC-MS analysis.

Cellular protein was determined using a protein assay kit with bovine serum albumin as a standard.

In vitro data analysis

The permeability (μL/mg protein) was expressed as the apical-to-basolateral side obtained by dividing the transported amount (μmol/mg protein) at the basolateral side by the initial concentration (μM) in the apical side. The permeability coefficient (P_{app}, cm/s) was calculated from the linear portion of an uptake vs time plot using the follow equation:

$$P_{app} = dQ/dt/A/C_0$$

where dQ/dt is the initial permeation rate across the Caco-2 cell monolayer (μmol/s), A is the surface area of the filter (cm^2), and C_0 is the initial concentration of the solution in the apical side (μM).

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Cell-to-medium ratio was obtained by dividing the cellular uptake amount by the concentration of test compound in the medium. Kinetic parameters for transport activity were estimated by nonlinear least-squares fitting of the data to the following equation using the MULTI program:

$$V = V_{max} \cdot S / (K_m + S) + K_d \cdot S$$

where V, S, K_m, V_{max}, and K_d represent the initial uptake rate, substrate concentration, Michaelis constant, maximum uptake rate, and first-order rate constant, respectively.

Pharmacokinetic study

The eight-week-old male rats (weights: 185-206g) were deprived of food for 12 hr before experiments. Oseltamivir was dissolved in distilled water, milk (commercially available cows' milk), Gly-Sar solution (20 mM or 125 mM), or casein solution (300 mg/10 mL), and was orally administered to rats at a single dose of 30 mg/kg (the dosing volume: 10 mL/kg). For the intravenous injection study, eight-week-old male rats (weights: 177-186g) were administered with 30 mg/kg of oseltamivir via the jugular vein (the dosing volume: 1 mL/kg). Blood samples were withdrawn from the jugular vein of rats with a heparinized syringe at designated times under anesthesia induced with diethyl ether. In the case of infant rats, one-week-old infant rats (weights: 16.5-20.6g) from the same parental female rat were used. Three of them (2 male, 1 female) were separated from the parental female rat and starved overnight, and the others (3 male, 2 female) remained with the parental rat and were fed milk. Oseltamivir dissolved in distilled water was orally administered to infant rats at a single dose of 30 mg/kg (the dosing volume: 10 mL/kg). Blood samples were collected from the jugular vein at 30 min after the start of administration under anesthesia induced with diethyl ether, and then the infant rats were decapitated. The brain was quickly excised, rinsed with ice-cold saline, blotted dry and weighed. Samples were stored at -30°C until analysis. Blood samples were

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centrifuged (1700 x g) for 15 min at 4°C to obtain plasma. As it has been reported that rat intestinal PEPT1 expression shows a diurnal rhythm, all in vivo studies have conducted at the same time of the day (Pan et al., 2004). Quantification of oseltamivir and Ro 64-0802 in plasma and brain tissues was performed using reported methods (Wiltshire et al., 2000) with some modification. Briefly, aliquots of brain tissues (100 mg) were homogenized with 1 mL of 5 mM ammonium acetate buffer, followed by centrifugation at 1700 x g, and 0.9 mL of the supernatant was subjected to solid-phase extraction (Empore Mixed Phase Cation, 7 mm/3 mL, 3M Bioanalytical Technologies, St. Paul, MN). The methods used for the extraction of plasma and brain homogenate were the same. The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were determined directly from the observed data. The area under the plasma concentration-time curve from time 0 to 6 hr ($AUC_{0\text{-}6hr}$) was estimated by the linear trapezoidal method.

Analytical methods

Aliquots (5 µL) of samples containing oseltamivir and Ro 64-0802 were injected into an HPLC system (LC-20A system, Shimadzu, Kyoto, Japan) equipped with a Capcellpak^{TM} OD column (150 x 2.0 mm i.d., Shiseido, Tokyo, Japan) using isocratic elution at 0.1 mL/min with 0.05% formic acid. Analytes were detected using a quadrupole mass spectrometer (LCMS-2010EV; Shimadzu, Kyoto, Japan) fitted with an electrospray ionization source. Analytes were detected in the positive mode, and protonated molecular ions at m/z=313 for oseltamivir and m/z=285 for Ro 64-0802 were monitored. Each value presented is the mean ± S.E.M of three samples. Statistical analysis was performed by means of Student's t test. A difference between means was considered to be significant when the P-value was less than 0.05.

RESULTS

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Characterization of oseltamivir transport across Caco-2 cell monolayers

We have shown that oseltamivir is a substrate of P-gp (Morimoto et al., 2008), and it was speculated that it might be a substrate of human carboxylesterase 1 (hCE-1, CES1A1, HU1) and/or carboxylesterase 2 (hCE-2, hiCE, HU3), which are present in several organs, including small intestine (Imai, 2006). Therefore, we first examined the effects of CES and P-gp on the permeability of oseltamivir across Caco-2 cell monolayers. Figure 1A shows the permeability coefficient of oseltamivir without BNPP pretreatment and absence of verapamil (○) was 0.45±0.02 x 10^{-6} cm/s (control value). The permeability coefficient of oseltamivir after pretreatment BNPP (▲) or in the presence of verapamil (■) was not showed significantly change (0.61±0.07 and 0.60±0.06 x 10^{-6} cm/s, respectively). The permeability of oseltamivir after pretreatment with BNPP and in the presence of verapamil (♦, 1.07±0.10 x 10^{-6} cm/s) showed the significantly higher than control value ($p<0.05$). Consequently, we decided that subsequent influx studies using Caco-2 cells should be done after preincubation with BNPP and after adding verapamil to the apical chamber. Figure 1B shows the inhibitory effects of dipeptide and temperature on oseltamivir permeability across Caco-2 cell monolayers. Gly-Sar and Trp-Gly, which are substrates of PEPT1, significantly decreased the permeability coefficient of oseltamivir to 0.39±0.07 and 0.49±0.04 x 10^{-6} cm/s, respectively from 0.61±0.04 x 10^{-6} cm/s (Fig. 1B). The permeability coefficient was greatly decreased to 0.12±0.01 x 10^{-6} cm/s at 4°C. Although we examined the concentration dependence of oseltamivir transport across Caco-2 cell monolayers, kinetic parameters could not be determined (data not shown).

Characterization of oseltamivir uptake by Caco-2 cells

The time course, concentration dependence, effect of PEPT1 substrates and temperature dependence of oseltamivir uptake by Caco-2 cells were studied and the results are shown in Figure 2 and Figure 3. The uptake of oseltamivir increased linearly up to 1 min (Fig. 2A). Thus, the initial

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uptake rate was obtained as the slope of the uptake over 1 min, and the incubation time of 1 min was used in subsequent studies. The concentration dependence of the initial uptake of oseltamivir was studied over the range from 30 μM to 10 mM (Fig. 2B). The uptake was saturable, and the K_m, V_{max}, and K_d values were 6.54 ± 2.03 mM, 45.6 ± 12.0 nmol/min/mg protein, and 0.470 ±0.517 μL/min/mg protein, respectively. The uptake was greatly reduced at 4°C. Both Gly-Sar and Trp-Gly significantly and concentration-dependently inhibited the uptake of oseltamivir (Fig. 3).

Characterization of oseltamivir uptake in HeLa/PEPT1

The uptake of oseltamivir by HeLa/PEPT1 increased in a time-dependent manner and was higher than that by HeLa/mock (Fig. 4A). Kinetic parameters of oseltamivir transport via PEPT1 were evaluated. The uptake of oseltamivir by PEPT1 was estimated after subtracting the uptake by HeLa/mock from those by HeLa/PEPT1. The uptake was saturable, and the K_m and the V_{max} values were estimated to be 8.59 ± 1.98 mM and 11.4 ± 1.68 nmol/10 min/mg protein, respectively (Fig. 4B). The uptake was markedly reduced when the temperature was lowered to 4°C (Fig. 5). In addition, the uptake of oseltamivir was decreased in the presence of PEPT1 substrates, 20 mM Gly-Sar or Trp-Gly (Fig. 5). On the other hand, the cell-to-medium ratio of Ro 64-0802, an active metabolite of oseltamivir, was 0.034±0.002 μL/10 min/mg protein, which was not significantly different from that of mock cells. This result suggested that the active metabolite is not a substrate of PEPT1.

Plasma concentration of oseltamivir in rats

Figure 6 shows the plasma concentration of unchanged drug after a single oral administration of oseltamivir (30 mg/kg) in rats (eight-week-old). The pharmacokinetic parameters of oseltamivir are summarized in Table 1. Coadministration of 20 mM Gly-Sar did not affect

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oseltamivir pharmacokinetics. However, when 125 mM Gly-Sar was administered concurrently, the plasma concentration of oseltamivir was dramatically reduced (Fig. 6), and the BA was decreased from 31.5% to 5.5%. When oseltamivir dissolved in milk was orally administered to rats, the plasma concentration of oseltamivir was decreased, and the BA was decreased to 11.7%. Concurrent administration of casein (300 mg/kg) also significantly decreased the plasma concentration of oseltamivir, and the BA was decreased to 5.5% (Table 1).

Plasma and brain concentrations of oseltamivir in pups

Oseltamivir were administered to fasting (non-breast-fed) rats, which were separated from the parental female rats overnight, and to non-fasting (breast-fed) rats born from the same mother. The plasma and brain concentrations of oseltamivir in fasting rats (13.4-15.3 μg/mL and 83.0-134 ng/g brain, respectively) were higher than those in non-fasting ones (0.344~0.884 μg/mL and 4.83~17.2 ng/g brain, respectively) after oral administration of the drug (Fig. 7). The plasma concentration was well correlated with the brain concentration.

DISCUSSION

We have already demonstrated that oseltamivir is transported by P-gp, and that the brain distribution is significantly affected by P-gp (Morimoto et al.,2008). During our study we noticed that the plasma concentration of oseltamivir, as well as its toxicity, was enhanced in fasted baby rats compared with non-fasted ones. Since baby rats are always fed milk, their gastrointestinal tract contains large amounts of di- and tripeptides, and we speculated that a peptide transporter might take part in the absorption of oseltamivir. Accordingly, we examined the intestinal transport of oseltamivir using Caco-2 cells and PEPT1-expressing HeLa cells. Initial uptake of oseltamivir by Caco-2 cells was saturable and temperature-dependent, and was inhibited by typical PEPT1

substrates (Fig. 2, Fig. 3). The transport properties of oseltamivir across a Caco-2 cell monolayer (Fig. 1) were consistent with the results of the uptake studies. These findings strongly suggested that oseltamivir is a substrate of PEPT1 and that its absorption is mediated, at least in part, by PEPT1. We directly confirmed that PEPT1 transports oseltamivir using PEPT1-expressing HeLa cell (Fig. 4, Fig. 5). The K_m value obtained in HeLa/PEPT1 cells was similar to that obtained in Caco-2 cells (Fig. 4). All these results suggested that the transport of oseltamivir across a Caco-2 cell monolayer was mediated by PEPT1. Interestingly, Ro 64-0802, which is an active metabolite of oseltamivir, was not a substrate of PEPT1, although the metabolite with a carboxylic acid moiety superficially seems to be more similar than oseltamivir to a dipeptide.

To estimate the effects of milk, and protein and peptides derived from milk, on absorption of oseltamivir, we conducted an in vivo study using eight-week-old and infant rats. Casein is a major protein in milk, and 300 mg/kg of casein is equivalent of 10 mL/kg of milk as protein content. Moreover, 1.25 mmol/kg (125 mM) of dipeptide is equivalent to 10 mL/kg of milk, if milk protein is completely digested to dipeptides. Therefore, we concurrently administered oseltamivir with 10 mL/kg of milk, 300 mg/kg of casein or 125 mM Gly-Sar to eight-week-old rats. Oseltamivir absorption was greatly reduced by these treatments (Fig. 6), suggesting that PEPT1 is indeed involved in gastric absorption of oseltamivir in rats. Such a peptide-drug interaction can directly affect the therapeutic efficacy and safety of substrate drugs, especially in infants, which are routinely fed milk. We then examined PEPT1-mediated peptide-drug interaction in one-week-old infant rats. The plasma and brain concentrations of oseltamivir were both higher in fasting rats than in non-fasting rats after oral administration of the drug (Fig. 7). These results suggest that milk peptides interacted with oseltamivir on PEPT1 and thereby inhibited absorption of oseltamivir in infant rats.

In this study, we demonstrated that oseltamivir is a substrate of PEPT1, and that this drug was absorbed at least in part via PEPT1 in small intestine. This result has two important implications.

In general, it is thought that intestinal absorption of ester-type prodrugs of carboxylic acids occurs via simple diffusion, and hence can be improved by increasing the lipophilicity. However, it now appears that PEPT1 may also play a role. This is consistent with previous reports on PEPT1-mediated transport of the L-valine ester prodrug valacyclovir (Balimane et al., 1998; Han et al., 1998; Sugawara et al., 2000). Absorption of various ester-type prodrugs might also be mediated by the influx transporter PEPT1. Secondly, the absorption of PEPT1 substrates might be influenced by eating, although so far there are few examples concerning the influence of food components on the absorption of medicines which are substrates of PEPT1. If other such peptide-drug interactions occur, they could directly affect the therapeutic efficacy and safety of substrate drugs, especially in infants. Further studies, including clinical studies, are needed.

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Footnotes

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FIGURE LEGENDS

Figure 1. Effects of inhibitors of carboxylesterase (CES), P-glycoprotein (P-gp) and PEPT1, and temperature on oseltamivir permeability across a Caco-2 cell monolayer

(A) Caco-2 cells were preincubated with 200 μM BNPP (♦,▲) or without BNPP (○, ■) for 40 min. Permeability of oseltamivir (100 μM) across a Caco-2 cell monolayer was measured in the absence (○,▲) and presence (♦,■) of verapamil (10 μM) at 37°C in HBSS at an apical-side pH of 6.0 and at a basolateral-side pH of 7.4.

Each point is the mean ± S.E.M. of three experiments. * $p<0.05$, significantly different from the control (●).

(B) Permeability coefficient of oseltamivir (100 μM) across a Caco-2 cell monolayer was measured at 37°C (control) or 4°C and in the presence of 10 mM Gly-Sar or 10 mM Trp-Gly at 37°C in HBSS at an apical-side pH of 6.0 and at a basolateral-side pH of 7.4.

Each column is the mean ±S.E.M. of three experiments. * $p<0.05$, significantly different from the control.

Figure 2. Time course (A) and concentration dependence (B) of oseltamivir uptake by Caco-2 cells

(A) Uptake of oseltamivir (100 μM) by Caco-2 cells was measured at 37°C in HBSS (pH 6.0).

Each point is the mean ±S.E.M. of three experiments.

(B) Uptake of oseltamivir by Caco-2 cells was measured for 1 min at 37°C and pH 6.0. The concentration of oseltamivir was 30 or 300 μM, or 1, 3, or 10 mM.

Each point is the mean ±S.E.M. of three experiments.

Figure 3. Effect of PEPT1 substrates and temperature on oseltamivir uptake by a Caco-2 cell

monolayer

Uptake of oseltamivir (100 μM) was measured for 1 min at 37°C or 4°C and pH 6.0. The effect of PEPT1 inhibitor Gly-Sar or Trp-Gly was examined at 37°C and pH 6.0.

Each column is the mean ±S.E.M. of three experiments. * $p<0.05$, significantly different from the control.

Figure 4. Time profile (A) and concentration dependence (B) of oseltamivir uptake by HeLa cells stably expressing PEPT1

(A) HeLa/PEPT1 (●) or mock (○) cells were incubated for the indicated periods at 37°C in HBSS (pH 6.0) containing 100 μM oseltamivir.

Each point is the mean ± S.E.M. of three experiments. * $p<0.05$, significantly different from the control.

(B) The uptake of oseltamivir by HeLa/PEPT1 (●) or mock (○) cells was determined at 10 min during incubation at 37°C and pH 6.0. The concentration of oseltamivir used was 30, 100 or 300 μM, or 1, 3, or 10 mM.

Each point is the mean ±S.E.M. of three experiments. * $p<0.05$, significantly different from the control.

Figure 5. Effect of PEPT1 substrate and temperature on oseltamivir uptake by HeLa cells stably expressing PEPT1

Uptake of oseltamivir (100 μM) by HeLa/PEPT1 cells was measured at 37°C or 4°C and in the presence of 20 mM Gly-Sar or 20 mM Tpr-Gly for 10 min at 37°C and pH 6.0. Each column is the mean±S.E.M. of three experiments. * $p<0.05$, significantly different from the control.

Figure 6. Plasma concentration of oseltamivir after oral administration to rats and inhibitory effect of peptides on oseltamivir absorption

Oseltamivir dissolved in distilled water (●), milk (○), 20 mM Gly-Sar solution (◊), 125 mM Gly-Sar solution (□) or 300 mg/10 mL casein solution (Δ) was orally administered (30 mg/kg).

Figure 7. Influence of peptides derived from milk on oseltamivir absorption in infant rats

Oseltamivir (30 mg/kg) was orally administered to one-week-old infant rats. Breast-fed male rats (□) and female rats (○) stayed with the parental female rat until shortly before the test. Nonbreast-fed male rats (■) and female rats (●) were separated from the parental female rat overnight. The line represents the correlation between plasma and brain concentrations: Brain concentration (ng/g brain) = 6.84 x Plasma concentration (μg/mL) + 6.26 ($R=0.975$, $p<0.05$).

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Table 1. Pharmacokinetics parameters of oseltamivir in rats [a)]

	C_{max} (μg/mL)	T_{max} (hr)	$AUC_{0\text{-}6hr}$ [b)] (μg·hr/mL)	Bioavailability
iv				
	—	—	8.07±0.63	1.000
po				
Distilled water	1.65±0.27	0.42±0.08	2.55±0.13	0.315
+Casein	—	—	0.45±0.01*	0.055
+20 mM Gly-Sar	1.65±0.33	0.42±0.08	2.82±0.23	0.349
+125 mM Gly-Sar	—	—	0.45±0.00*	0.055
Milk	0.31±0.04*	0.67±0.17*	0.94±0.07*	0.117

a) Oseltamivir (30 mg/kg) was intravenously (iv) or orally (po) administered to male rats.

b) Area under the plasma concentration curve from 0 to 6 hr after a single administration.

, Significantly different from rats to which oseltamivir dissolved in distilled water was orally administered ($P<0.05$).

Each value, except bioavailability, is the mean ±S.E.M. of three animals.

Fig. 1

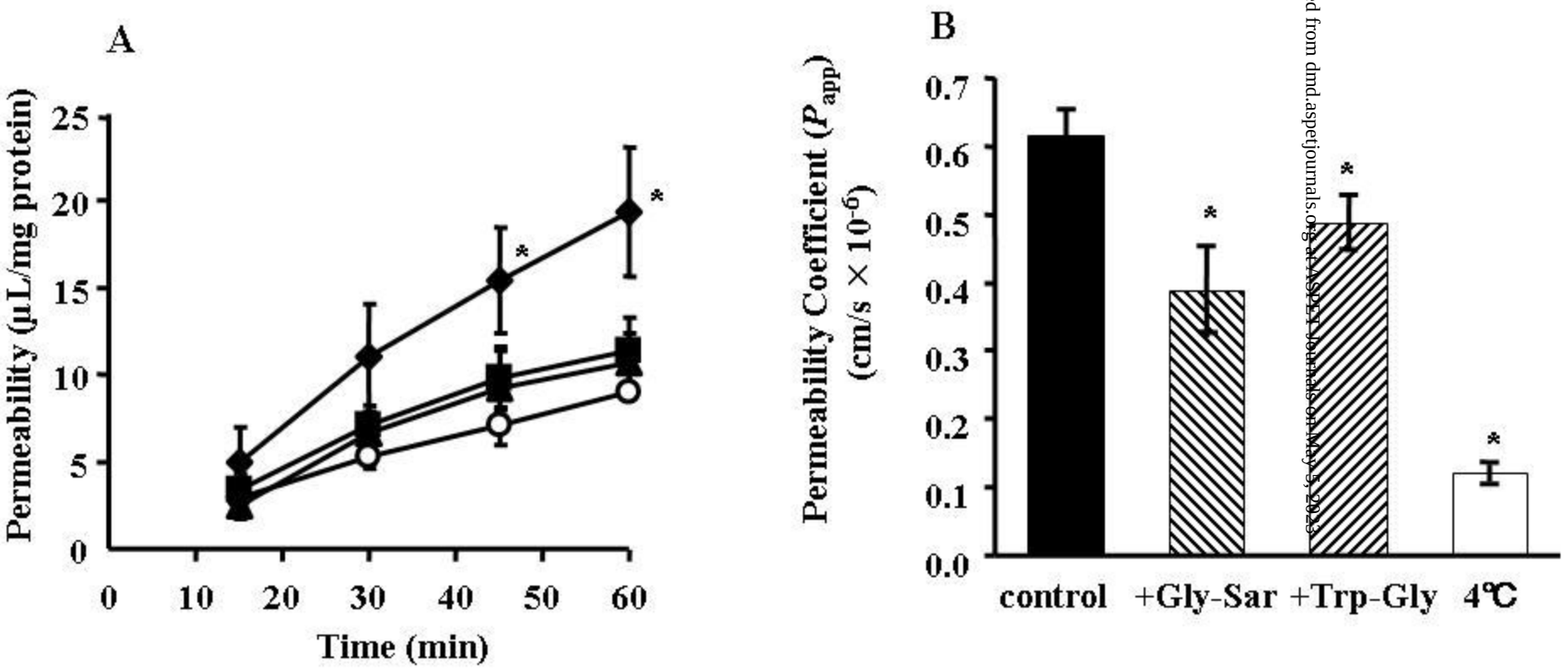

Fig. 2

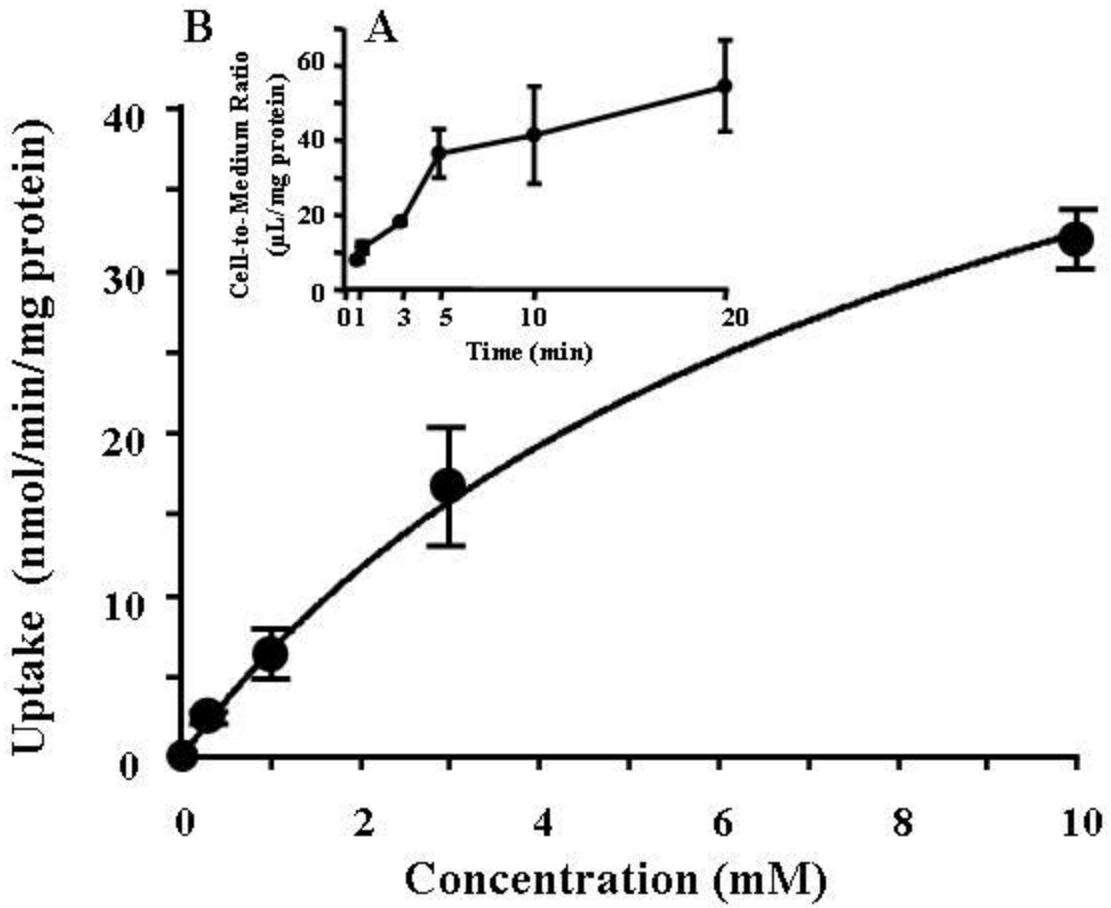

Fig. 3

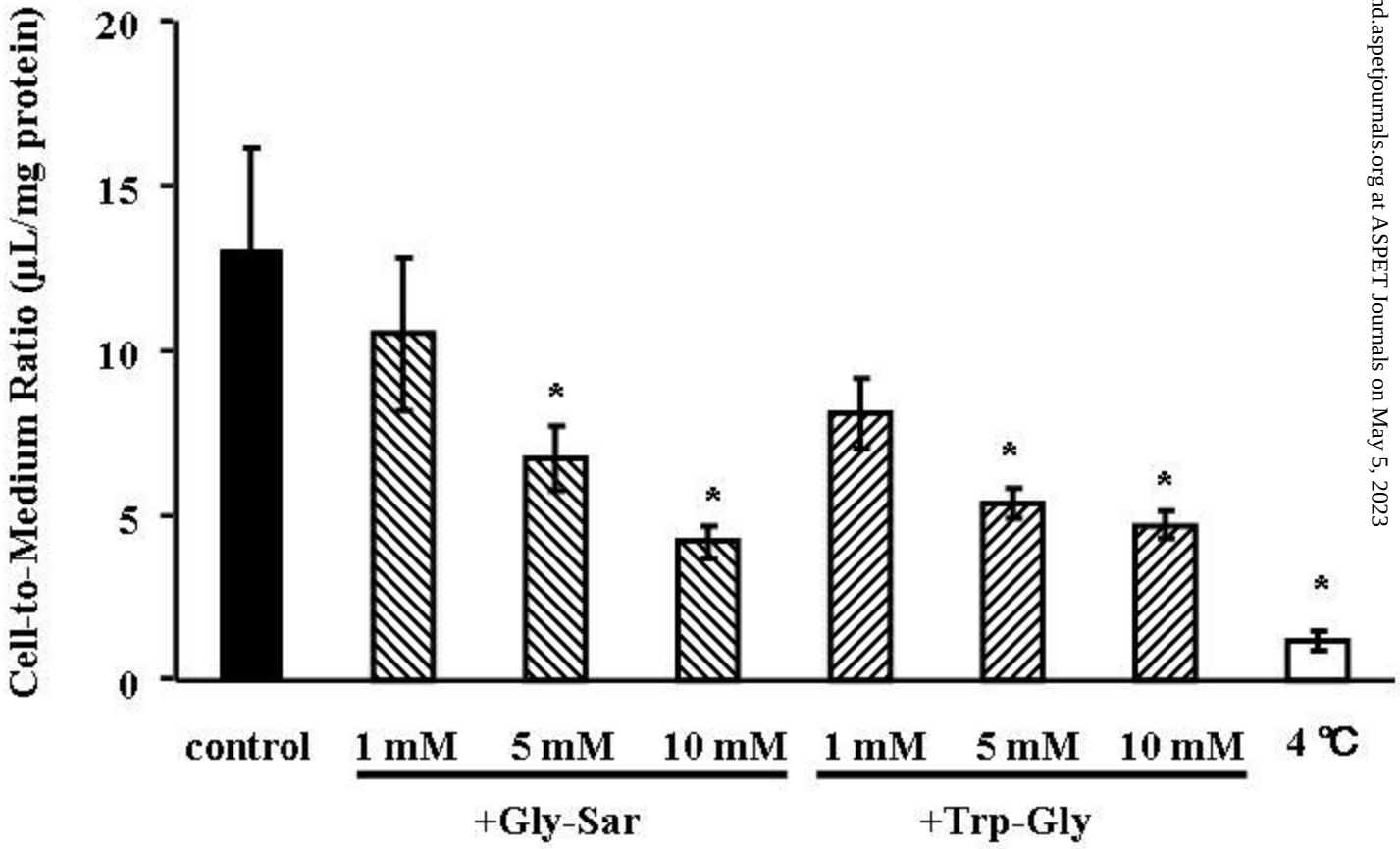

Fig. 4

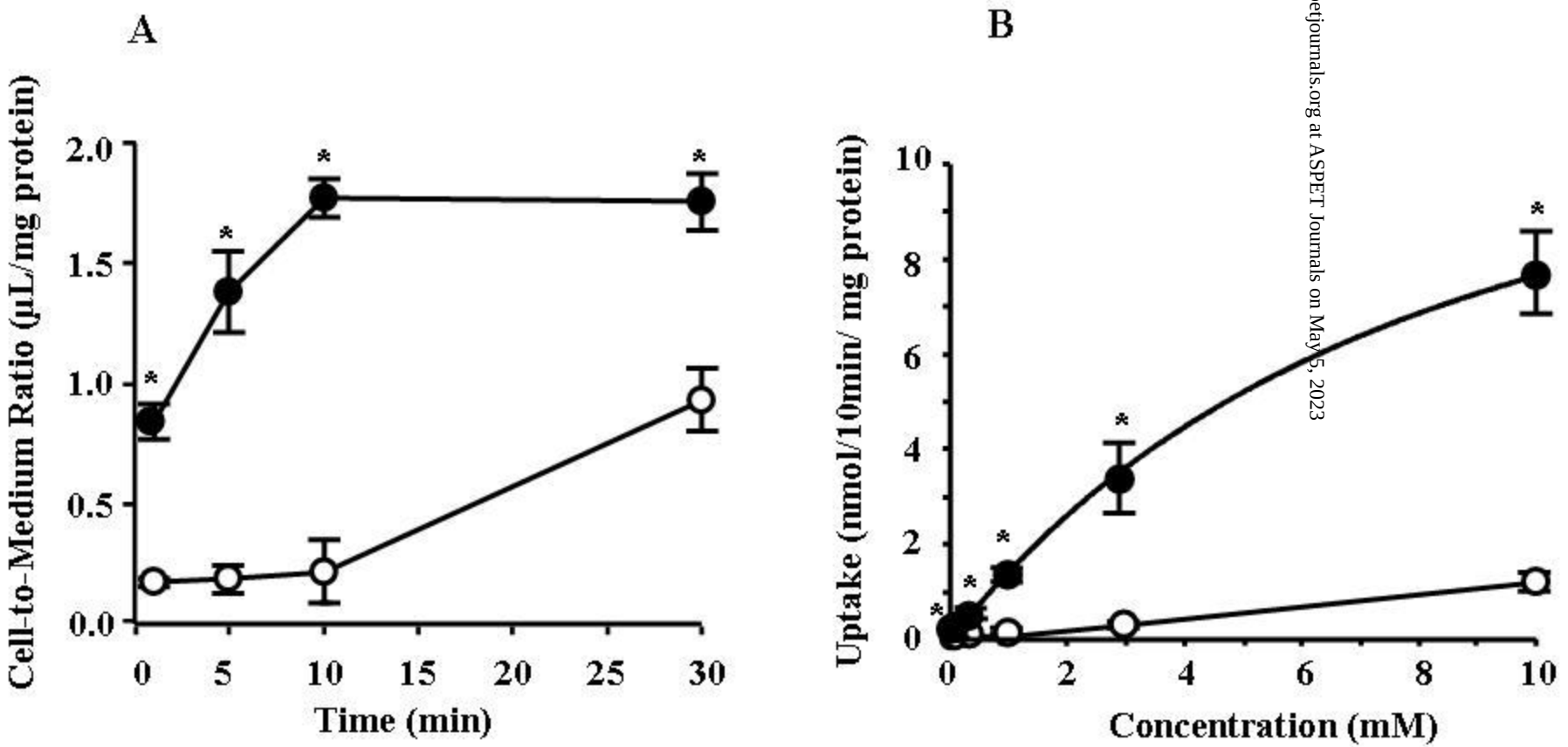

Fig. 5

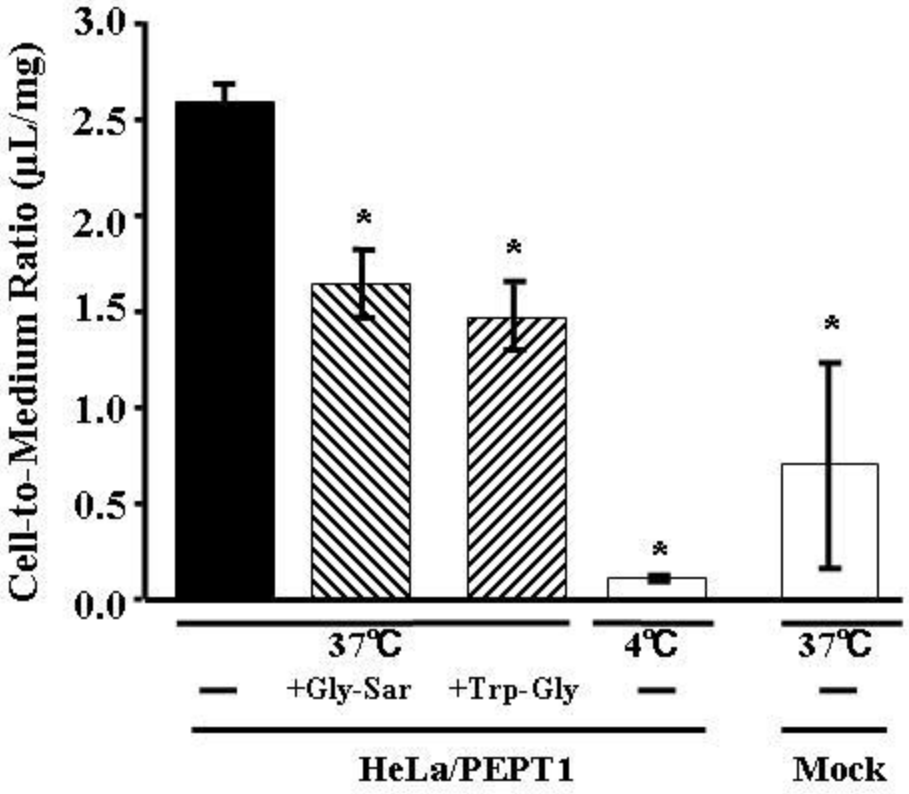

Fig. 6

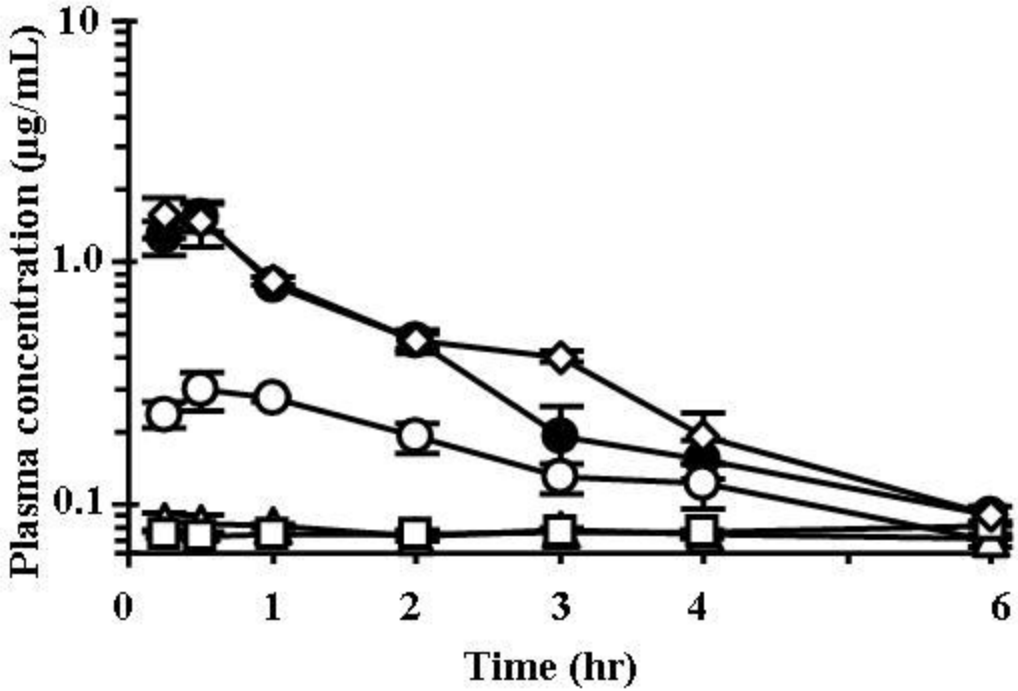

Fig. 7